A STUDY OF BINDING PROPERTIES OF THE ISOLECTINS FROM THE *DOLICHOS BIFLORUS* PLANT USING AFFINITY ELECTROPHORESIS

Carl BORREBAECK and Marilynn E. ETZLER

Department of Biochemistry and Biophysics, University of California, Davis, CA 95616, USA

Received 16 June 1980

1. Introduction

The lectin in the seeds of the *Dolichos biflorus* plant specifically agglutinates type A erythrocytes. This ability is due to the fact that it recognizes and binds to terminal non-reducing α-*N*-acetyl-galactosamine, the immunodominant carbohydrate of blood group A substance [1,2]. The *Dolichos biflorus* lectin is a glycoprotein and can be fractionated into two electrophoretically distinguishable forms called the A and B forms [3].

This study presents a further investigation of the binding properties of the two isolectins using affinity electrophoresis which is a combination of affinity chromatography and conventional electrophoresis. Continuous polyacrylamide tube affinity electrophoresis [4,5] and flatbed agarose affinity electrophoresis [6] have so far been used to study various properties of glycoproteins and lectins. We have developed a discontinuous non-denaturing polyacrylamide tube affinity electrophoretic system that gives an environment in which the lectin better retains its binding ability. Using this system more accurate information concerning binding properties, association constants and state of aggregation can be achieved.

2. Materials and methods

2.1. *Purification of the lectin*

The *Dolichos biflorus* lectin was isolated as in [1,3] by adsorption of the protein onto insoluble polyleucyl hog blood group A+H substance [7] and specific elution with 0.01 M *N*-acetyl-D-galactosamine. The ligand was removed by molecular exclusion chromatography and the lectin was then concentrated by ultrafiltration in a Diaflo ultrafiltration apparatus equipped with a PM-10 filter.

2.2. *Fractionation of the lectin into forms A and B*

As described [3], the lectin was separated into 7 fractions based upon its elution profile from an affinity column of concanavalin A immobilized to (con A–) Sepharose. Using this approach the first peak (fraction I–III) which was the unretarded protein, was enriched in form B, whereas the fractions (IV–VII) which were eluted with a concave gradient of methyl α-D-glucopyranoside contained increasingly greater proportions of form A [3].

2.3. *Affinity electrophoresis*

This system was developed on the basis of existing non-affinity approaches [9,10] to give an electrophoretic environment in which the isolectins could fully retain their binding capacities. The following solutions were used in the preparation of the gels:

Solution A (pH 7.5): 48 ml 1 M HCl, 6.85 g Tris and 0.46 ml TEMED, up to 100 ml with H_2O.

Solution B (pH 5.5): 41.8 ml 1 M H_3PO_4, 4.85 g Tris and 0.46 ml TEMED, up to 100 ml with H_2O.

Solution C: 20.0 g acrylamide and 0.525 g bisacrylamide, up to 100 ml with H_2O.

Solution D: 10.0 g acrylamide and 2.5 g bisacrylamide, up to 100 ml with H_2O.

Solution E: 140 mg ammoniumpersulfate up to 100 ml with H_2O.

Solution F: 56 mg ammoniumpersulfate up to 10 ml with H_2O.

Tubes (6 mm diam.) were filled up to a height of 100 mm with running gel solution which was prepared by combining 1 part A, 2 parts C, 1 part H_2O and then adding 4 parts of E. This running gel is 5% polyacryl-

amide and maintains a running pH below 8.0. After polymerization of the running gels, a stacking gel solution consisting of 1 part B, 2 parts D, 4 parts H_2O and 1 part F was added so that the combined heights of the running and stacking gels were ~110 mm.

The gels were then placed in a chamber containing an electrode buffer of 12 mM imidazole and 43 mM diethylbarbituric acid (pH 7.0). The samples (25 μg protein in 20% glycerol, bromophenol blue was used as tracking dye) were stacked for 1 h at 1 mA/tube and then the current was increased to 3 mA/tube for 3 h. The electrode buffer was recirculated during the run to maintain a constant pH in the buffer reservoirs. Immediately following electrophoresis the gels were fixed for 30 min in trichloracetic acid (12.5%) and then stained for 4 h in Coomassie brilliant blue [11].

The above described non-denaturing polyacrylamide gels were easily converted to affinity gels by addition of hog blood group A+H substance to solution A before polymerization takes place. A final concentration of 0.5 mg entrapped blood group substance/ml of running gel was used. Different amounts of free ligand (final conc. 0–50 mM), i.e., *N*-acetyl-D-galactosamine, were also added to solution A before polymerization to obtain a gel containing both entrapped as well as free ligand.

2.4. *Preparation of* N-*acetyl-D-galactosamine immobilized to Sepharose*

The carbohydrate was coupled to epoxyactivated Sepharose (Pharmacia Fine Chemicals, Uppsala) as in [12]. This procedure employs a spacer arm between the resin and the carbohydrate and thereby helps to avoid steric hindrance. However, the circumstances under which the coupling takes place have been shown to deacetylate the C-2 position in certain acetylated amino sugars to a small extent [13]. Therefore, after immobilization, the sugar hapten was reacetylated [14] to achieve an affinity gel with maximum capacity.

3. Results and discussion

The *N*-acetyl-D-galactosamine Sepharose prepared as above was capable of binding soy bean agglutinin at a capacity of 0.5 mg lectin/ml Sepharose; however the *Dolichos biflorus* lectin did not bind to the resin (fig. 1). Since > 90% of the bisoxirane coupling of this sugar takes place through the C-6 hydroxyl group of the carbohydrate, this result indicates that the *Dolichos biflorus* lectin requires an unsubstituted hydroxyl at the C-6 position. This restriction would be in addition to the already established requirement of the lectin for an unsubstituted acetamido group at the C-2 position of the sugar [1,2].

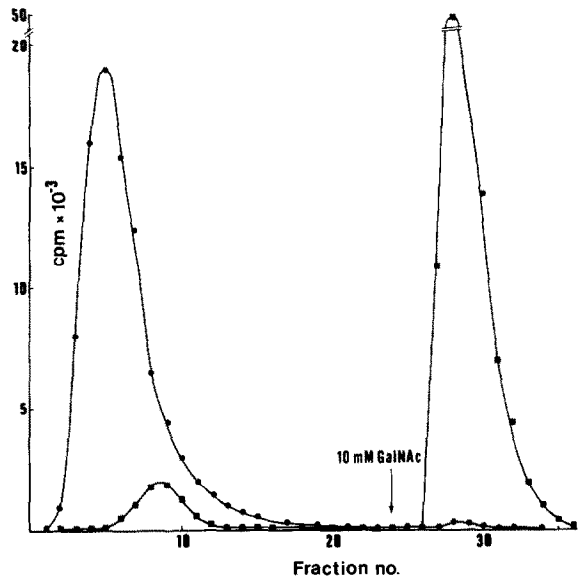

Fig. 1. Affinity chromatogram of *Dolichos biflorus* lectin (•—•) and soy bean agglutin (▪—▪) using *N*-acetyl-D-galactosamine, immobilized onto expoxy-activated Sepharose, as a ligand. *N*-acetyl-D-galactosamine was used at 10 mM as eluting medium. The specific activities of the two lectins are about the same.

The failure of the lectin to bind to the above resin excluded the use of the epoxyactivation method to prepare ligand–polysaccharide complexes for entrapment in affinity electrophoresis gels. To avoid time consuming chemical immobilization techniques of the ligand we designed a system in which the high molecular weight blood group A+H substance was entrapped in the polyacrylamide network of the gels. The *Dolichos biflorus* lectin was run in this system and the results were evaluated according to calculations based on the work in [15] concerning quantitative affinity chromatography. Electrophoretic mobility has in our case been inserted instead of elution volumes to give the equation:

$$\frac{l}{l_0 - l} = \frac{K_i}{c_i}\left(1 + \frac{c}{K}\right) \qquad (1)$$

where c = concentration of free ligand in the gel; c_i = concentration of entrapped ligand in the gel; l = elec-

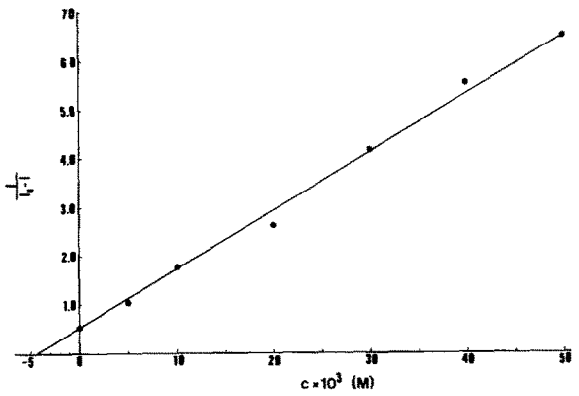

Fig. 2. An affinity electrophoretic plot utilizing eq. (1). The interception of the abscissa gives the dissociation constant between the lectin and the free ligand. K_d for unfractionated lectin was 4.3×10^{-3} M.

trophoretic mobility of the lectin at a given c and c_i; l_0 = electrophoretic mobility of the lecitin at $c_i = 0$; K_i = dissociation constant between the lectin and the entrapped ligand; and K = dissociation constant between the lectin and the free ligand.

K is given directly from the equation above when $l/l_0 - l$ equals zero, i.e., $K = -c$. The assumptions under which eq. (1) is valid have been extensively discussed [16]. Furthermore, affinity electrophoresis does not give sufficient information for determination of number of binding sites in a protein but can distinguish between mono- vs multivalent interactions as judged by the appearance of the slopes [16,17].

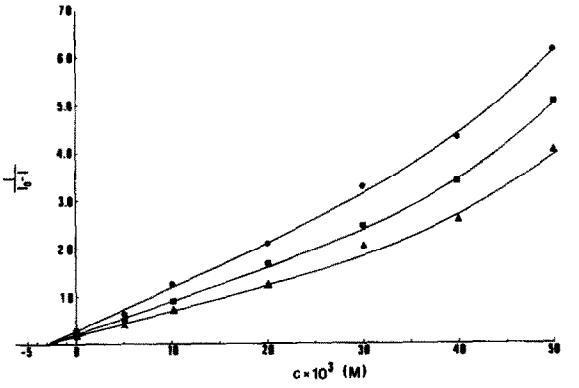

Fig. 3. Affinity electrophoretic plots of fractions containing various amounts of forms A and B of the *Dolichos biflorus* lectin. Note the curvilinear slopes that indicate multivalent interactions between the isolectins and the entrapped ligand. K_d between the isolectins and the free ligand are the same and estimated to be 2.8×10^{-3} M. (▲—▲) Fraction II (enriched in form B; (■—■) fraction IV (enriched in form A); (●—●) fraction VII (predominantly form A).

Using the above equation, the association constant between the *Dolichos biflorus* lectin and the free ligand, *N*-acetyl-D-galactosamine, has been determined to be 2.2×10^2 M^{-1} (fig.2). This lectin was a mixture of the two isolectins, form A and B, described [3].

Fig.3 shows the results of affinity electrophoresis using lectin fractions (II, IV, VII) enriched in forms A or B that were obtained by affinity chromatography on con. A–Sepharose [3]. Although the association constants of the 3 fractions for *N*-acetyl-D-galactosamine are essentially identical, the slopes of the plots vary significantly. The slope of the curve obtained for fraction II, which is enriched in form B, is lower than the slope obtained with fraction IV which is greatly enriched in form A. The greatest slope was, however, obtained with fraction VII which is predominantly form A. The difference in slope among the 3 fractions indicates that forms A and B of the lectin have different association constants for the entrapped blood group substance. The slight curvatures of the plots are characteristic of bi- and multivalent interactions between a protein and its ligand [16,17]. It therefore appears that the 2 isolectins may differ in their extent of aggregation or in the accessibility of their combining sites to the entrapped ligand.

The above data demonstrate the adaptability of affinity electrophoresis at a pH where most biological components fully retain their binding capacity and when chemical immobilization of the ligand may be unfavorable. Furthermore, by changing the concentration of the entrapped ligand and using a monovalent fraction of the lectin it would be possible to estimate the bonus effect, due to multipoint interactions, that the lectin possesses. The assessment of such contributions of multivalency to the binding strengths of proteins will probably be fundamental to the understanding of lectin–ligand interactions in plants as well as of immunological interactions in vertebrates.

Acknowledgements

This work was supported by NIH grant GM 21882 and USDA grant SEA 5901–0–0242.

References

[1] Etzler, M. E. and Kabat, E. A. (1970) Biochemistry 9, 869–877.

[2] Hammarström, S., Murphy, L. A., Goldstein, I. J. and Etzler, M. E. (1977) Biochemistry 16, 2750–2755.
[3] Carter, W. G. and Etzler, M. E. (1975) J. Biol. Chem. 250, 2756–2762.
[4] Horejsi, V., Ticha, M. and Kocourek, J. (1977) Biochim. Biophys. Acta 499, 301–308.
[5] Kerckaert, J. P., Bayard, B. and Biserte, G. (1979) Biochim. Biophys. Acta 576, 99–108.
[6] Bog-Hansen, T. C. (1973) Anal. Biochem. 54, 480–488.
[7] Kaplan, M. E. and Kabat, E. A. (1966) J. Exp. Med. 123, 1061–1081.
[8] Cuatrecasas, P. and Parikh, I. (1972) Biochemistry 11, 2291–2299.
[9] Davis, B. J. (1964) Ann. NY Acad. Sci. 121, 405–427.
[10] Williams, D. E. and Reisfeld, R. A. (1964) Ann. NY Acad. Sci. 121, 373–381.
[11] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[12] Vretblad, P. (1976) Biochim. Biophys. Acta 434, 169–176.
[13] Uy, R. and Vold, F. (1977) Anal. Biochem. 81, 98–107.
[14] Levvy, G. A. and McAllen, A. (1959) Biochem. J. 73, 127–132.
[15] Dunn, B. M. and Chaiken, I. M. (1977) Biochemistry 14, 2343–2349.
[16] Horejsi, V. (1979). In: Proc. Affinity Chromatography and Molecular Interactions (Egly, J. M. ed) vol. 86, pp. 391–398, INSERM, Paris.
[17] Eilat, D. and Chaiken, I. M. (1979) Biochemistry 18, 790–795.